Identification of a conserved residue responsible for the autoinhibition of cGMP-dependent protein kinase Iα and β

Keizo Yuasa[a], Hideo Michibata[a], Kenji Omori[a], Noriyuki Yanaka[b,*]

[a] *Discovery Research Laboratory, Tanabe Seiyaku Co. Ltd., 2-50, Kawagishi 2-chome, Toda, Saitama 335-8505, Japan*
[b] *Discovery Research Laboratory, Tanabe Seiyaku Co. Ltd., 16-89 Kashima 3-chome, Yodogawa-ku, Osaka 532-8505, Japan*

Received 9 November 1999; received in revised form 21 December 1999

Edited by Shozo Yamamoto

Abstract **We isolated a constitutively active form of cGMP-dependent protein kinase Iα (cGK Iα) by PCR-driven random mutagenesis. The replacement of Ile-63 by Thr in the autoinhibitory domain results in the enhancement of autophosphorylation and the basal kinase activity in the absence of cGMP. The hydrophobicity at position 63 is essential for the inactive state of cGK Iα, and Ile-78 of cGK Iβ is also required for the autoinhibitory property. Furthermore, cGK Iα (Ile-63-Thr) is constitutively active in vivo. These findings suggest that a conserved residue in the autoinhibitory domain was involved in the autoinhibition of both cGK Is.**

1. Introduction

An increase in intracellular cGMP levels results in smooth muscle relaxation, platelet aggregation, intestinal secretion and endochondral ossification mediated through the activation of cGMP-dependent protein kinases (cGKs) [1–3]. Two major isoforms of cGK, termed cGK I and cGK II, were identified to be encoded by distinct genes, and cGK I has two isoforms, designated Iα and Iβ, produced by alternative mRNA splicing [4]. cGK Iα and cGK Iβ were demonstrated to be dimeric enzymes that contain several domains with well defined functions. The regulatory compartment located in the N-terminal region contains the leucine zipper motif, multiple autophosphorylation sites and an autoinhibitory domain, followed by two cGMP-binding and catalytic domains [5,6]. The primary structure of the two alternative isoforms differs only in the N-terminal ~100 residues, sharing 36% identity in this region. While removal of the leucine zipper motif along with the autoinhibitory region (residues 1–93) of cGK Iβ results in a monomeric and constitutively active enzyme [5], cGK Iβ lacking the leucine zipper motif (residues 1–61) is still activated in the presence of cGMP [7]. Both cGK Iα and Iβ are supposed to contain substrate sites and a putative pseudosubstrate sequence within their autoinhibitory domain, suggesting that these regions interact with the catalytic site and inhibit phosphotransferase activity in the absence of cGMP. In cAMP-dependent protein kinase (cAK), two tandem basic amino acids in the autoinhibitory region are important for recognition as the consensus phosphorylation sequence and for potent inhibition of the catalytic subunit of cAK by the regulatory subunit [8]. S.H. Francis and colleagues have demonstrated that Arg-75 at the pseudosubstrate site of cGK Iβ is required for autoinhibition [9], and that the level of phosphorylation of Ser-79 located within this sequence significantly increases in the presence of cGMP and correlates with an increase in basal kinase activity [10]. In fact, a recent study on the pseudosubstrate sequence of cGK Iβ showed that replacement of Arg-75 by Ala, or of Ser-79 by an acidic residue, aspartic acid, to mimic the effect of autophosphorylation, generates strong basal kinase activity [11]. However, this basic residue Arg-75 is replaced by Ala in the pseudosubstrate sequence ^{59}RAQGISAEP of the cGK Iα, and Ser-64 in cGK Iα corresponding to Ser-79 is reported to be only a minor phosphoacceptor site [12]. Thus, the amino acid residue responsible for autoinhibition of cGK Iα is less well understood. To determine the amino acid residues critical for autoinhibition and function of this enzyme, the N-terminal sequence of cGK Iα was subjected to PCR-driven random mutagenesis. Here, we isolated a constitutively active enzyme of cGK Iα, and identified a conserved residue in the autoinhibitory domain, which was involved in the autoinhibitory mechanism of both cGK Iα and cGK Iβ.

2. Materials and methods

2.1. Materials

Restriction endonucleases and DNA-modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan). COS-7 cells were from Dainippon Pharmaceutical (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FCS) were obtained from Life Technologies. [γ-^{32}P]ATP was a product of Amersham Pharmacia Biotech.

2.2. Plasmid construction

The full-length bovine cGK Iα cDNA was a gift from Dr. Thomas M. Lincoln (The University of Alabama at Birmingham). A cDNA encoding full-length cGK Iα was subcloned into the *Eco*RI and *Kpn*I sites of the expression vector pFLAG-CMV-2 (Kodak) fused in frame with the epitope sequence for anti-FLAG antibody (pFLAG-cGK Iα). The cDNA clone that encodes the full-length human cardiac troponin I (cTnI) was provided by Dr. Yuichiro Maeda, and subcloned into the *Not*I site of the expression vector pFLAG-CMV-2. To introduce random mutations in cGK Iα sequence, a PCR was run for 35 cycles (1 min at 94°C, 2 min at 50°C and 3 min at 72°C) under standard conditions using a set of PCR primers (5′-AAACATGAGC-GAGCTGGAGGAAGAC-3′, 5′-CCCTGAGAATGGTCCAGAGC-TCTCCAC-3′) and the full-length bovine cGK Iα cDNA as a temperate, and the PCR products were digested with *Eco*RI and *Mlu*I. Excised 1.1 kb cDNAs harboring the PCR-generated mutations were ligated into *Eco*RI and *Mlu*I sites of the pFLAG-cGK Iα. The full-length human cGK Iβ cDNA was obtained by standard PCR protocol, and subcloned into pFLAG-CMV-2 as we described previously [13]. Site-directed mutagenesis was performed using Quick-

*Corresponding author. Fax: (81)-6-6300 2593.
E-mail: n-yanaka@tanabe.co.jp

Abbreviations: cGK, cGMP-dependent protein kinase; cAK, cAMP-dependent protein kinase; cTnI, cardiac troponin I

0014-5793/00/$20.00
PII: S0014-5793(99)01786-X

change™ site-directed mutagenesis kit (Stratagene) according to the protocol of the manufacturer. In each case, the mutation was confirmed by DNA sequencing analysis.

2.3. In vitro kinase assay

COS-7 cells were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO_2. The full-length bovine cGK Iα or human cGK Iβ cDNA, and the full-length human cTnI cDNA in the expression vector pFLAG-CMV-2 were transiently expressed in COS-7 cells [13] using LipofectAMINE PLUS according to the manufacturer's instructions (Life Technologies). Cells were washed with ice-cold phophate-buffered saline 24 h after transfection, and scraped in an ice-cold TNE buffer (10 mM Tris–HCl at pH 7.5, 1% NP40, 0.15 M NaCl, 1 mM EDTA, 10 mg/ml aprotinin, 10 mM leupeptin and 1 mM dithiothreitol). Cell extracts were centrifuged at 16 000×*g* for 15 min at 4°C to remove cellular debris. The supernatants were immunoprecipitated with anti-FLAG M5 monoclonal antibody (Kodak) with protein G-Sepharose overnight at 4°C by rotation. The beads were washed five times with TNE buffer. The in vitro kinase reaction was performed in kinase reaction buffer (50 mM Tris–HCl at pH 7.5, 20 mM magnesium acetate, 0.2 mM [γ-^{32}P]ATP, 2 μM protein kinase A inhibitor (5-24) (PKI (5-24), Calbiochem), 5 mM glycerophosphoric acid and 1 mM sodium orthovanadate) in the presence or absence of cGMP (5 μM final concentration). The samples were incubated at 30°C for 30 min and centrifuged at 16 000×*g* and 4°C. The beads were mixed with an equal volume of 2×sodium dodecyl sulfate (SDS) sample buffer and heated at 95°C for 5 min, and the denatured proteins were subjected to SDS–polyacrylamide gel electrophoresis (PAGE). In order to evaluate the relative cGK I-dependent kinase activity in the presence and absence of cGMP, in vitro kinase reaction was performed in kinase reaction buffer using the synthetic peptide BPDEtide (Calbiochem) as a substrate. The values were corrected relative to the kinase activity of wild-type cGK Iα in the absence of cGMP taken as one, and are expressed as the mean of three independent experiments. For the immunoblotting analysis, the proteins separated by SDS–PAGE were transferred onto polyvinylidene difluoride membranes (Millipore). After being blocked with Block Ace (Snow Brand Milk Products) overnight, the membrane was incubated with anti-FLAG M5 monoclonal antibody for 2 h at room temperature. Bound primary antibody was detected using horseradish peroxidase-conjugated anti-mouse IgG (Sigma) and visualized by the ECL Western blotting detection system (Amersham Pharmacia Biotech).

2.4. In vivo kinase assay

COS-7 cells were co-transfected with pFLAG-cGK Iα and pFLAG-cTnI as described above. For [^{32}P]phosphate labeling, transiently transfected COS-7 cells were washed and preincubated with phosphate-free medium containing 1 mCi/ml [^{32}P]phosphate (500 μCi/ml, NEN Life Science Products) for 3 h, and either left untreated or treated with 8-(4-chlorophenylthio) guanosine-3′,5′-monophosphate (8-CPT-cGMP, Sigma) for 30 min. Cells were scraped in an ice-cold cell extract buffer (10 mM Tris–HCl at pH 7.5, 0.1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 10 mg/ml aprotinin, 10 mM leupeptin, 5 mM glycerophosphoric acid and 1 mM sodium orthovanadate). The cell extracts were centrifuged at 16 000×*g* for 15 min at 4°C to remove cellular debris, and the supernatants were immunoprecipitated with anti-FLAG M5 monoclonal antibody with protein G-Sepharose for 4 h at 4°C by rotation. The beads were washed twice with cell extract buffer, and immune complexes were eluted by heating at 95°C in 2×SDS sample buffer, subjected to SDS–PAGE and autoradiographed at −80°C.

Table 1
Phosphorylation of BPDEtide by cGK Iα in vitro

		Relative kinase activity		Ratio (plus/minus of cGMP)
		cGMP minus	cGMP plus	
Iα	I (WT)	1.00	5.98 ± 0.01	5.98
	G	2.85 ± 0.21	3.65 ± 0.19	1.28
	A	1.80 ± 0.17	3.80 ± 0.05	2.11
	T	1.92 ± 0.07	3.93 ± 0.21	2.05
	S	3.16 ± 0.21	3.48 ± 0.20	1.10
	L	0.86 ± 0.07	2.62 ± 0.06	3.06
	F	0.88 ± 0.04	4.49 ± 0.03	5.08
	V	0.92 ± 0.14	6.00 ± 0.23	6.53
Iβ	I (WT)	0.22 ± 0.03	2.95 ± 0.07	13.72
	G	1.40 ± 0.04	2.46 ± 0.09	1.76

The effects of several other amino acids substituted for Ile-63 on the basal kinase activity were tested by measuring the basal and cGMP-activated kinase activities using BPDEtide, as a substrate. COS-7 cells were transfected with each expression plasmid for FLAG-cGK Iα. Whole cell lysates were immunoprecipitated with anti-FLAG antibody, and subjected to an in vitro kinase assay. Reactions were performed in the absence (minus) or presence (plus) of 5 μM cGMP.

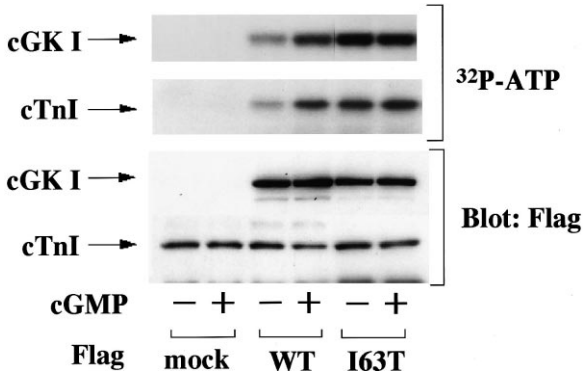

Fig. 1. Phosphorylation of cTnI by cGK Iα in vitro. COS-7 cells were transfected with the expression plasmid for FLAG-cTnI. Where indicated, plasmid expressing either FLAG-cGK Iα or FLAG-cGK Iα (I63T) was included in the transfection. Whole cell lysates were immunoprecipitated with anti-FLAG antibody (IP: Flag), and cGK activity was measured in an in vitro kinase assay. Reactions were performed in the absence (−) or presence (+) of 5 μM cGMP. To monitor the expression level of each kinase, the immunoprecipitates were blotted with anti-FLAG antibody (Blot: Flag).

3. Results

We attempted to isolate constitutively active forms of cGK Iα by PCR-mediated mutagenesis [14]. The mutated FLAG-tagged cGK Iα were expressed in COS-7 cells, and immunoprecipitates were subjected to a kinase assay using FLAG-tagged cTnI as a substrate. We isolated one clone (HM clone) showing enhanced basal kinase activity and autophosphorylation in the absence of cGMP. Sequence analysis revealed two point mutations, Arg-17→Gly (R17G) and Ile-63→Thr (I63T), in the N-terminal region of the cGK Iα sequence. To clarify which of these mutations was responsible for the constitutive activation, we constructed single mutants (cGK Iα (R17G) and cGK Iα (I63T)), and investigated their kinase activity. Replacement of Arg-17 by Gly did not alter the basal activity of cGK Iα (data not shown), whereas replacement of Ile-63 by Thr generated significant increases in the level of basal kinase activity and autophosphorylation when compared with wild-type cGK Iα (Fig. 1). The protein expression level of the constitutively active mutant was similar to that of wild-type cGK Iα (Fig. 1). Next, we tested the effects of several other amino acids substituted for Ile-63 on the basal kinase activity. The effects of these mutants were tested by measuring the basal and cGMP-activated kinase activities using BPDEtide, as a substrate. The protein expression levels of these mutants were similar to that of wild-type cGK Iα (data

not shown). Substitution of Ile-63 by Gly, Ala or Ser increased the basal kinase activity, being accompanied by a relatively low ratio of kinase activity in the presence and absence of cGMP. Interestingly, replacement of Ile-63 by hydrophobic residues such as Phe or Val resulted in a similar enzymatic property as to that of the wild-type cGK Iα with increases in kinase activity of 5.1- and 6.5-fold, respectively (Table 1). Furthermore, immunocytochemical studies revealed that cGK Iα (I63T) localized to cytoplasm but not to the nucleus in the presence and absence of cGMP (data not shown), being consistent with recent observations by S.P. Collins et al. [11].

Both cGK Iα and cGK Iβ contain putative pseudosubstrate sites in their autoinhibitory regions. The pseudosubstrate site resembling the consensus phosphorylation sequence is supposed to play an important role in the interaction between the autoinhibitory and catalytic regions. The pseudosubstrate sites of cGK Iα and cGK Iβ are ^{59}RAQGISAEP and ^{74}KRQAISAEP, respectively. Because the Ile-63 in cGK Iα is conserved in cGK Iβ at position 78, we constructed the same mutant of cGK Iβ (I78G). Similarly, we transfected cGK Iβ (I78G) into COS-7 cells, and subjected the immunocomplex to in vitro kinase assay. As we expected, the replacement of Ile-78 by Gly results in an upregulation of autophosphorylation and basal kinase activity in the absence of cGMP (Fig. 2), indicating that cGK Iβ (I78G) is a constitutively active mutant.

Next, to determine whether cGK Iα (I63T) is constitutively active in intact cells, cGK Iα and cTnI were co-expressed as FLAG-tagged proteins in COS-7 cells. After ^{32}P-labeling, the cells were untreated or treated with a membrane-permeable cGMP analogue, 8-CPT-cGMP. Immunoprecipitation by using anti-Flag antibody showed that cTnI was significantly phosphorylated by cGK Iα (I63T) in the absence of 8-CPT-cGMP (Fig. 3). A kinase-defective mutant of cGK Iα, cGK Iα (D501A), did not catalyze the phosphorylation of cTnI, confirming that cTnI is phosphorylated by exogenously produced cGK Iα in vivo. These observations showed that cGK Iα (I63T) is constitutively active not only in vitro but in intact cells, suggesting the availability of this mutant as a tool for in vivo analysis. Transgenic mouse expressing the constitutively active form of cGK I will provide the important informations for understanding of cGMP-mediated signaling pathways in vivo.

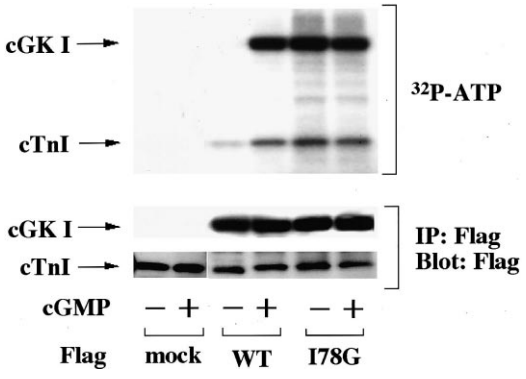

Fig. 2. Phosphorylation of cTnI by cGK Iβ in vitro. COS-7 cells were transfected with expression plasmid for FLAG-cTnI. Where indicated, plasmid expressing either FLAG-cGK Iβ or FLAG-cGK Iβ (I78G) was included in the transfection. Whole cell lysates were immunoprecipitated with anti-FLAG antibody (IP: Flag), and cGK activity was measured in an in vitro kinase assay. Reactions were performed in the absence (−) or presence (+) of 5 μM cGMP. To monitor the expression level of each kinase, the immunoprecipitates were blotted with anti-FLAG antibody (Blot: Flag).

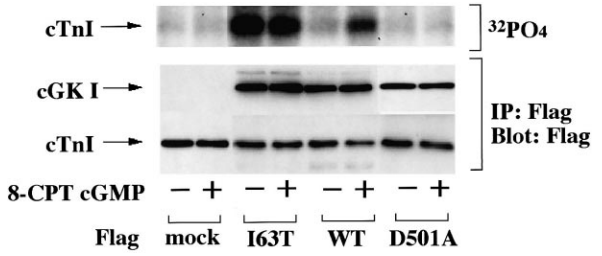

Fig. 3. Phosphorylation of cTnI by cGK Iα in vivo. COS-7 cells were co-transfected with expression vectors for either FLAG-cGK Iα, FLAG-cGK Iα (I63T) or kinase-defective mutant of FLAG-cGK Iα (cGK Iα D501A) in combination with the expression plasmid for FLAG-cTnI. Cells were labeled with ^{32}P as detailed under Section 2. ^{32}P-labeled cell lysates were immunoprecipitated with anti-FLAG antibody (IP: Flag), and the immunoprecipitates were subjected to SDS–PAGE and autoradiography. To monitor the expression levels of kinase and cTnI proteins, the immunoprecipitates were blotted with anti-FLAG antibody (Blot: Flag).

4. Discussion

cGK Iα and cGK Iβ contain a putative pseudosubstrate site, ^{59}RAQGISAEP and ^{74}KRQAISAEP, within their autoinhibitory regions, respectively. The underlined amino acid is thought to occupy the phosphate acceptor site (P0) according to the consensus substrate sequence (RRXSX) in the type II regulatory subunit (R subunit) of cAK. The pseudosubstrate site is supposed to tightly interact with the catalytic site, and inhibit the kinase activity in the absence of cGMP. The association of cGMP with cGMP-binding domains of cGK Is is suggested to relieve autoinhibition of the catalytic site. Previous studies have demonstrated that the binding of cGMP resulted in a conformational change in cGK Is [15,16]. The binding of cGMP to cGK Iα or cGK Iβ caused a large electronegative charge shift of the enzyme on ion exchange chromatography. Additionally, in cGK Iβ, activation by cGMP decreased mobility on native gel electrophoresis. As noted also, a previous report has suggested that enhanced proteolysis within and near the pseudosubstrate site by cGMP is due to increased solvent exposure of the autoinhibitory region in the presence of cGMP. A basic residue, Arg-75 located at the P−2 position, is known to play an important role in the close interaction between the autoinhibitory sequence and catalytic domain in the inactive cGK Iβ [9]. In fact, replacement of Arg-75 by Ala generates strong basal kinase activity [11]. The current study indicated that the hydrophobic residue at the P+1 position is also a critical determinant for potent autoinhibition of catalysis and autophosphorylation of cGK Is in the absence of cGMP. Our results with cGK Is may be applicable to cGK II, because the hydrophobic residue (Ile or Val) at the P+1 position is well conserved among cGKs. The effect of the hydrophobicity of the pseudosubstrate sequence on the inhibition of kinase activity was previously tested using synthetic peptides. The heat-stable inhibitor protein of cAK (PKI) is a potent, competitive inhibitor of the catalytic subunit of this enzyme, and contains a pseudosubstrate sequence. The structure of the cAK catalytic subunit (C subunit) co-

crystallized with PKI has demonstrated that Leu-24 at the P+1 position in PKI stably associates with the hydrophobic pocket formed by Leu-198, Pro-202 and Leu-205 in the C subunit [17]. In fact, a previous report showed that substitution at the P+1 position with Gly significantly increased the K_i value from 3 nM to 420 nM, indicating that Leu-24 is important for the high-affinity binding of the inhibitor peptide [18]. Additionally, a recent report on RI subunit of cAK demonstrated that the inhibitory effect was significantly reduced when Ile-98 at the P+1 position was replaced by Ala [8]. These observations strongly supported the importance of the hydrophobicity at the P+1 position of the pseudosubstrate sequence in cGK Is for inhibition of basal kinase activity and of autophosphorylation in the absence of cGMP.

As previously reported, autophosphorylation increased the sensitivity of cGK Iβ to activation by cGMP [10]. In cGK Iβ, the increase in basal kinase activity in the presence of cGMP closely correlates with autophosphorylation at Ser-79. Also, a recent report has shown that replacement of Ser-79 by an acidic residue to mimic the effect of autophosphorylation produces strong basal kinase activity [11]. However, in cGK Iα, autophosphorylation is observed at Thr-59, not at Ser-65 which corresponds to Ser-79 in cGK Iβ, when incubated in the presence of cGMP. The constitutively active cGK Iα described in this study could enhance basal activity in the absence of cGMP, the effect being accompanied by an increase in the autophosphorylation. Further study is needed to determine the phosphorylated amino acids in this constitutively active cGK Iα, providing information about the mechanism underlying activation of cGK Iα.

Acknowledgements: We thank Drs. T.M. Lincoln and Y. Maeda for generously donating cDNAs. We also thank Dr. H. Sakurai for helpful discussion.

References

[1] Lohmann, S.M., Vaandrager, A.B., Smolenski, A., Walter, U. and De Jonge, H.R. (1997) Trends Biochem. Sci. 22, 307–312.
[2] Pfeifer, A., Klatt, P., Massberg, S., Ny, L., Sausbier, M., Hirneiβ, C., Wang, G.-X., Korth, M., Aszódi, A., Andersson, K.-E., Krombach, F., Mayerhofer, A., Ruth, P., Fässler, R. and Hofmann, F. (1998) EMBO J. 17, 3045–3051.
[3] Pfeifer, A., Aszódi, A., Seidler, U., Ruth, P., Hofmann, F. and Fässler, R. (1996) Science 274, 2082–2086.
[4] Wernet, W., Flockerzi, V. and Hofmann, F. (1989) FEBS Lett. 251, 191–196.
[5] Francis, S.H. and Corbin, J.D. (1994) Adv. Pharmacol. 26, 115–170.
[6] Francis, S.H. and Corbin, J.D. (1994) Annu. Rev. Physiol. 56, 237–272.
[7] Wolfe, L., Francis, S.H. and Corbin, J.D. (1989) J. Biol. Chem. 264, 4157–4162.
[8] Poteet-Smith, C.E., Shabb, J.B., Francis, S.H. and Corbin, J.D. (1997) J. Biol. Chem. 272, 379–388.
[9] Francis, S.H., Smith, J.A., Colbran, J.L., Grimes, K., Walsh, K.A., Kumar, S. and Corbin, J.D. (1996) J. Biol. Chem. 271, 20748–20755.
[10] Smith, J.A., Francis, S.H., Walsh, K.A., Kumar, S. and Corbin, J.D. (1996) J. Biol. Chem. 271, 20756–20762.
[11] Collins, S.P. and Uhler, M.D. (1999) J. Biol. Chem. 274, 8391–8404.
[12] Aitken, A., Hemmings, B.A. and Hofmann, F. (1984) Biochim. Biophys. Acta 790, 219–225.
[13] Yuasa, K., Michibata, H., Omori, K. and Yanaka, N. (1999) J. Biol. Chem. (in press).
[14] Onishi, M., Nosaka, T., Misawa, K., Mui, A.L.-F., Gorman, D., Mcmahon, M., Miyajima, A. and Kitamura, T. (1998) Mol. Cell. Biol. 18, 3871–3879.
[15] Chu, D.-M., Corbin, J.D., Grimes, K.A. and Francis, S.H. (1997) J. Biol. Chem. 272, 31922–31928.
[16] Zhao, J., Trewhella, J., Corbin, J., Francis, S., Mitchell, R., Brushia, R. and Walsh, D. (1997) J. Biol. Chem. 272, 31929–31936.
[17] Kemp, B.E., Parker, M.W., Hu, S., Tiganis, T. and House, C. (1994) Trends Biochem. Sci. 19, 440–444.
[18] Glass, D.B., Cheng, H.C., Mende-Mueller, L., Reed, J. and Walsh, D.A. (1989) J. Biol. Chem. 264, 8802–8810.